PB81-192007

Stimulation of Oxidant Production in Alveolar Macrophages by Pollutant and Latex Particles

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

27 Dec 78

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TECHNICAL REPORT DATA (Please read Instructions on the reverse before completing)		MS-78-081
1. REPORT NO. EPA-600/J-80-260	2. JOURNAL ARTICLE	3. PB81-192007
4. TITLE AND SUBTITLE Stimulation of Oxidant Production in Alveolar Macrophages by Pollutant and Latex Particles		5. REPORT DATE April 1981 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Gary E. Hatch, Donald E. Gardner, and Daniel B. Menzel		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Inhalation Toxicology Branch, ETD Health Effects Research Lab US EPA Research Triangle Park, NC 27711		10. PROGRAM ELEMENT NO. AAGA1D 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Office of Research and Development (RTP,NC) Health Effects Research Laboratory US Environmental Protection Agency Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES Published In: Environmental Research 23: 121-136, 1980		

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

PRODUCED BY:
NATIONAL TECHNICAL INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report) UNCLASSIFIED	21. NO. OF PAGES
RELEASE TO PUBLIC	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE

PB5-178 081

ENVIRONMENTAL RESEARCH 23, 121–136 (1980)

EPA-600/J-80-260
Journal Article

Stimulation of Oxidant Production in Alveolar Macrophages by Pollutant and Latex Particles[1]

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Received December 27, 1978

Air pollutant dusts as well as chemically defined particles were examined for their activating effect on oxidant production (O_2^- and H_2O_2) in guinea pig alveolar macrophages (AM). Oxidant production was measured as chemiluminescence of albumin-bound luminol. All particles examined stimulated the AM in a dose-dependent manner to different maximal levels of oxidant production. Amphibole asbestos samples were the most active of all agents studied. Various immune reactants as well as silica, metal-oxide-coated fly ash, polymethyl methacrylate beads, and chrysotile asbestos had intermediate activity, while fugitive dusts, polybead carboxylate microspheres, glass and latex beads, uncoated fly ash, and fiberglass had the lowest activity. In addition to direct stimulatory action on AM, particles also lowered the subsequent responsiveness of the cells to the bacterial peptide stimulant, *N*-formylmethionyl phenylalanine. This effect was only partially due to the cytotoxicity of the particles. While some relationship appeared to exist between stimulatory activity and cytotoxicity of the particles, the exact role of O_2^- in mediating cytotoxic effects is still open to question. Oxidant production in AM is a parameter which may be important in determining the pathological effects of particles as well as of materials adsorbed to their surfaces.

INTRODUCTION

Some environmental stresses may cause lung disease through alteration of immunological mechanisms. Overactivity of these mechanisms would cause inflammatory damage to lung tissue, while underactivity would lead to infection. A series of investigations conducted in our laboratory have shown that some pollutants decrease the activity of host-defense mechanisms in the lung leading to increased susceptibility to infection in experimental animals (Gardner and Graham, 1977). The present report deals with stimulatory effects of particulate pollutants on pulmonary alveolar macrophages (AM), which might result in inflammatory damage.

Oversecretion of lysosomal enzymes by AM has been postulated as a cause of tissue degradation in emphysema (Ackerman and Beebe, 1974). In addition to lysosomal enzymes, AM secrete powerful oxidant radicals which may contribute to inflammatory damage (Fridovich, 1978). These radicals appear to function normally in the killing of invading parasites (Babior, 1978). Most studies on oxidant production in phagocytes have been with blood leukocytes or peritoneal exu-

[1] This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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date cells, although AM are probably exposed to a greater quantity and diversity of particulate agents than the former cell types. An early observation by Sbarra and Karnovsky (1959) was that leukocytes undergo a rapid increase in cyanide-resistant respiration during the ingestion of starch and latex particles. A large part of this increment in oxygen consumption has since been shown to be due to the activation of an oxidase located in the plasma membrane or granules of the phagocyte (Iverson *et al.*, 1977; Briggs *et al.*, 1975). A variety of particles and membrane-reactive agents, as well as specific immunological mediators, are capable of activating this oxidase, leading to an increased production of superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) (Goldstein *et al.*, 1975; Weiss *et al.*, 1978; Root and Metcalf, 1977).

Different types of particles vary considerably in their *in vivo* effects. Silica and asbestos, for example, have been shown to produce inflammatory exudates, fibrosis, and, under some conditions, neoplasms, while latex, TiO_2, and carbon particles are almost inactive in these respects (Allison, 1976; Hamilton *et al.*, 1976; Wagner *et al.*, 1976; vanWaarde *et al.*, 1977; Zaidi, 1969). The effects of a variety of particles on *in vitro* oxidant production by AM have been examined here in an attempt to define another parameter in macrophage activation which may be related to *in vivo* effects of these particles. The assay of AM oxidant production is based on our previous studies using the chemiluminescent oxidation of albumin-bound luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Allen and Loose, 1976; Hatch *et al.*, 1978). The sensitivity of this detection system makes possible the measurement of oxidants produced by alveolar macrophages stimulated with low concentrations of particules or with *N*-formylmethionyl peptides. In the present study we describe the stimulatory effects of various particulate and fibrous air pollutants, and plastic and glass microspheres on oxidant production in guinea pig AM. An ancillary purpose of the study has been to determine what role, if any, the macrophage oxidants play in mediating the cytotoxic effects of the particles.

MATERIALS AND METHODS

Particles and Fibers

Asbestos samples (UICC standard amosite, anthophyllite, crocidolite, Rhodesian chrysotile A, and Canadian chrysotile B) were used. The percentage of these asbestos samples which would be inhaled to the alveolar level of the lung has been reported previously and ranges from 67 to 87% by weight (Timbrell and Rendall, 1972). Microscopic examination indicated that most (~ 98%) fibers had a width of less than 1 μm and a length of less than 10 μm.

Fly ash particles were prepared by the Fine Particles Section of the Illinois Institute of Technology Research Institute, Chicago, Illinois, from a fly ash sample provided by the Environmental Protection Agency (Aranyi *et al.*, 1977). This ash was obtained by electrostatic precipitation of coal smoke. The fly ash was fractionated by air classification (Bahco Microparticle Classifier) into three particle size ranges (<2, 2–5, and 5–8 μm). Metal oxide coatings of the fly ash were applied by precipitation of the metal hydroxides in aqueous solution. After filtration and washing, the particles were heated to 675°C to convert the metal hydroxides into the respective oxides. Atomic absorption spectrophotometry indicated

that the uncoated fly ash contained <0.03% Pb, <0.03% Ni, and <0.01% Mn, while the coated fly ash of all sizes contained between 2.63 and 3.85% by weight of the metal oxide specified. Little variation was seen in the metal oxide content of different size particles.

Road dust, used here as a typical fugitive dust sample, was fine air cleaner test dust (No. 1-1543094-121, AC Spark Plug Div., General Motors Corp., Flint, Mich.). This is a fugitive dust from Arizona which has been used as a standard for testing air filters. It contains mostly silicate materials, and 80% by weight of the dust is composed of particles of less than 5-μm diameter. Rock dust was obtained from the rock-crusher bag house of a local hot-mix asphalt plant. This dust consisted mostly of α-quartz (60%) and sodium aluminum silicate (30%), as determined by X-ray diffraction. The particle size distribution, determined by Coulter counter and Anderson three-stage cyclone, showed it to be composed mostly (95% by weight) of particles of less than 5-μm diameter. Fiberglass was a homogenized sample of commercially available rolled home insulation. Fibers were 2–5 μm in diameter and 30–100 μm in length, and some nonfibrous material was present.

Latex particles were obtained from Dow Chemical, Midland, Michigan. The large latex particles (7.6 μm) were polystyrene divinylbenzene, while the smaller were polystyrene. Fumed silica (0.014 μm), *N*-formylmethionyl phenylalanine (F-Met-Phe), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), superoxide dismutase (SOD), and zymosan were obtained from Sigma Chemical Company, St. Louis, Missouri. Silicic acid (325 mesh) was obtained from Fisher Scientific. Glass beads (3–8 μm), methacrylate (0.32 μm), and carboxylate (1.44 μm) microspheres were obtained from Polysciences, Inc., Warrington, Pennsylvania.

Samples of particles and fibers were suspended in 0.85% saline to a concentration of 10 mg/ml and stored at 4°C. Zymosan (unopsonized) was stored frozen. All particle solutions were shaken vigorously before use. F-Met-Phe-coated latex and glass beads were prepared by incubating the beads in a 0.1 mM solution of F-Met-Phe in neutralized saline for 3 hr at 25°C, then washing the beads twice by centrifugation in saline. Possible effects of agents dissolved in the supernatants of the particles were determined after filtration of the particles through a 0.22-μm-pore-size Millipore filter (Millipore Corp., Bedford, Mass.). Filtrates were examined both for a stimulatory effect on the macrophages and for an inhibitory effect on chemiluminescence induced by the peptide stimulant, F-Met-Phe. Direct light quenching effects of the particles were determined by comparing the F-Met-Phe-induced chemiluminescent response seen immediately after addition of the particles with that seen in the absence of particles.

Preparation of Alveolar Macrophages

Male Hartley-strain guinea pigs (Cesarean obtained, barrier sustained, 650–800 g) were obtained from The Charles River Breeding Laboratories, Wilmington, Mass. The technique of Coffin *et al.* (1968) was used to obtain AM. Animals were killed by cervical dislocation, the trachea was exposed, incised, and a catheter inserted. The lungs were flushed five times with 10 ml of saline warmed to 37°C. Cellular suspensions were made in Geys balanced salt solution (GBSS, Microbiological Associates, Bethesda, Md.).

Measurement of Oxidant Production by Macrophages

Oxidant production in cell suspensions was measured as the light intensity resulting from the oxidation of luminol as described previously (Hatch *et al.*, 1978). Samples of AM were prepared by adding one part of cellular suspension to one part of 1% bovine serum albumin (BSA) in GBSS containing 0.2 mM luminol. The luminol–BSA solution was prepared by stirring luminol and BSA (Pentax crystallized bovine albumin, Miles Laboratories, Inc., Miles Research Products, Elkart, Ind.) in GBSS at room temperature until dissolved (4 h). The solution was sterilized by micropore filtration and stored in a dark bottle at 4°C. Chemiluminescence due to oxidation of luminol by the macrophages was quantitated using an ATP spectrophotometer (Model 3000, SAI Technology Co., San Diego, Calif.). Light intensity was monitored continuously by use of a strip chart recorder (Fisher Recordall Series 500, Pittsburgh, Pa.) and expressed in the arbitrary units of volts per million cells. The temperature of the counting chamber of the spectrophotometer was 31°C; therefore, vials containing cells were kept in a water bath maintained at the same temperature. New polyethylene scintillation vials (Fischer Scientific Co.) or 30-mm Falcon petri dishes were used, depending on the experiment. The AM were found to attach about equally to both types of containers; however, those attached to the polyethylene vials generally showed a larger chemiluminescent response than those attached to the petri dishes. Cells were allowed to incubate for 1 h before particles were added.

Measurement of macrophage viability

Cells were incubated overnight (16 hr) in GBSS containing 0.5% BSA, 50 units penicillin G/ml, and 50 μg streptomycin/ml. Viability was determined either by the response of the cells to 10 μM F-Met-Phe or by 0.4% trypan blue exclusion. A decline in the F-Met-Phe response with time after addition of particles was investigated as an indirect measurement of cell death based on our previous finding that nonviable cells do not respond to peptides. Trypan blue staining was performed after first suspending attached cells with a rubber policeman.

RESULTS

All types of particles examined caused stimulation of oxidant production by AM which was measurable as luminol chemiluminescence. In accord with previous findings, the stimulation by particles followed a more prolonged time course than that produced by soluble peptides, such as F-Met-Phe (Hatch *et al.*, 1978). Peak levels of chemiluminescence were reached 2–3 min after stimulation with the peptides and between 10 and 120 min after addition of particles. After peak levels were reached, a gradual return to basal levels occurred which usually required at least twice as long as the initial rise to peak levels. The maximal intensity as well as the time course of the chemiluminescent response to the particles was quite reproducible within a given batch of cells. While considerably more variation was seen between cells from different animals, the effects of the particles relative to each other appeared to follow a definite pattern. Representative dose–response studies of the different types of particles which comprise Figs. 1 through 3 were repeated on at least 3 separate days with similar results.

Effects of the size of latex microspheres on macrophage oxidant production. All sizes of latex microspheres caused a dose-dependent increase in macrophage oxidant production except the 7.6-μm particles which only showed a slight upward trend. Figure 1 shows the peak chemiluminescent intensity (per million cells) seen following the addition of latex of different diameters, plotted against the concentration of latex expressed in milligrams per milliliter in the cell suspension. The larger microspheres (7.6 μm) had only a negligible effect at 1 mg/ml concentrations, while the same concentration of microspheres of diameters between 0.1 and 3.2 μm approximately doubled the chemiluminescence seen in control cells. The trend of an increasing chemiluminescent response with decreasing particle diameters was seen in all experiments performed. No consistent differences were noted between the time courses of response induced by the large particles and those of the smaller particles. None of the supernatants obtained by filtration of the latex suspensions had a stimulatory effect on the cells.

Effects of asbestos on macrophage oxidant production. The three types of amphibole asbestos—crocidolite, anthophyllite, and amosite—were extremely active in stimulating luminol oxidation by AM, while the serpentine forms (chrysotiles A and B) were about one-fourth as active. The same relative stimulatory activity of all types of asbestos (crocidolite > amosite, anthophyllite >>

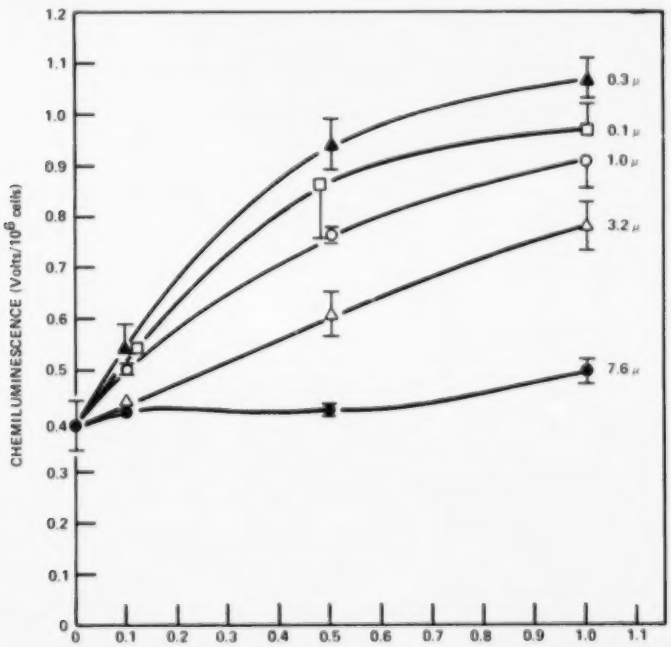

FIG. 1. Dose–effect relationship of different size latex particles on oxidant production in alveolar macrophages. Each value represents the mean ± one-half the range of the maximal level of chemiluminescence seen after addition of the latex particles. Sizes of latex are noted on the figure.

chrysotiles A and B) was seen in all experiments performed. Maximum levels of chemiluminsecence were often reached by low concentrations (0.4 mg/ml) of asbestos (Fig. 2). As the concentration of asbestos was increased above the maximum, a decrease was seen in the time required to reach the maximal response, as well as the time required to return to basal levels. Fiberglass insulation, which was the only other fibrous material studied, had almost no stimulatory activity for AM.

Effect of fly ash on macrophage oxidant production. Lead and nickel oxide coating of the fly ash increased the maximum chemiluminescence as much as sixfold over the uncoated fly ash, and over the manganese oxide-coated particles which also give very low responses (Fig. 3). The relative ranking of the increases in chemiluminescence ($PbO > NiO > MnO_2$ and uncoated) was the same in all experiments performed. The smaller particles were usually slightly more active on a weight percentage basis than the large particles; however, a reversal of this trend was consistently seen at the higher doses of the PbO-coated particles. It will be seen later that the effects of the fly ash may be altered due to substances present in the supernatant of the particles, rather than to the particles themselves. None of the supernatants had stimulatory activity by themselves; however, they did enhance the stimulation caused by the peptide, F-Met-Phe.

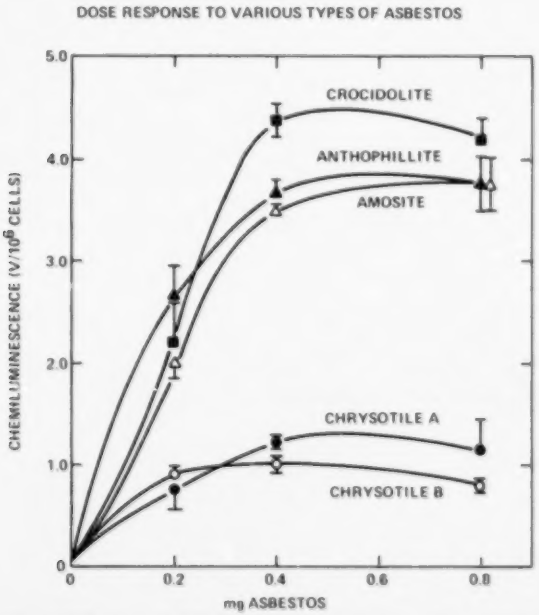

FIG. 2. Dose–effect relationship of different types of UICC asbestos fiber on oxidant production by guinea pig alveolar macrophages. Each value represents the mean ± SD in three replicate vials of the maximum level of chemiluminescence seen after addition of the asbestos. Types of asbestos fiber are noted on the figure.

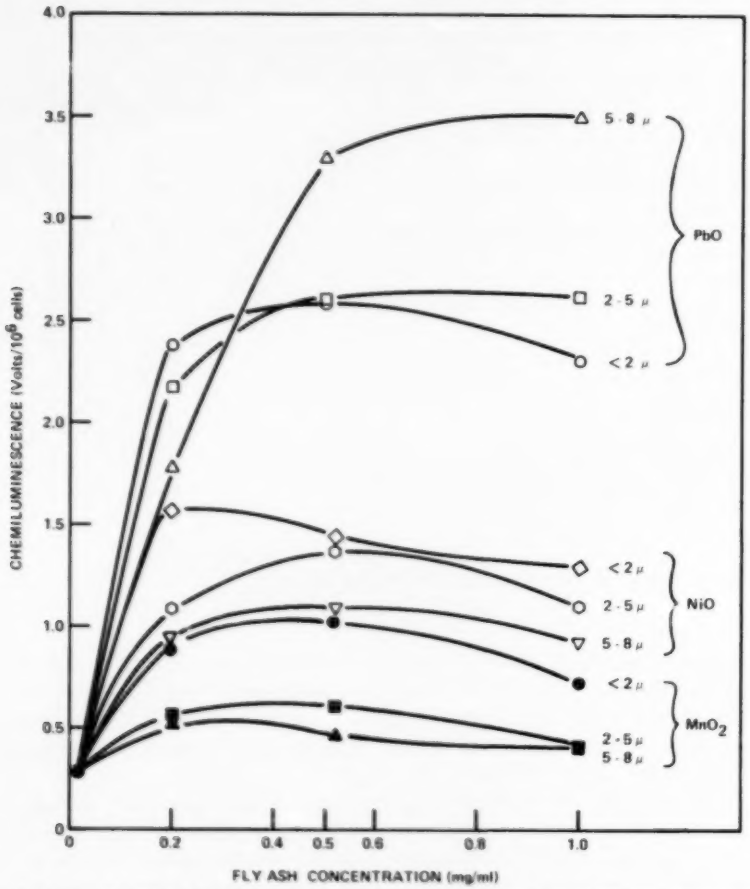

FIG. 3. Dose–effect relationship of metal oxide-coated fly ash samples of different sizes on oxidant production by alveolar macrophages. Sizes and metal oxide coating of the fly ash are indicated on the figures. Dose–response curves for the uncoated fly ash of all sizes were superimposable on the 2- to 5- and 5- to 8-μm MnO_2-coated fly ash dose–response curves.

Comparison of stimulatory effects of various particles. Table 1 shows a comparison of the effects of all particles we have tested. Crocidolite (and other amphibole asbestos samples not shown) stimulated oxidant production to the highest maximal levels. The next most active agent, F-Met-Phe-coated latex, was only about one-half as active as the amphibole asbestos samples, and F-Met-Phe-treated latex was almost four times more active than untreated latex. Fumed silica, F-Met-Phe-treated glass beads, silicic acid, zymosan, polymethyl methacrylate microspheres, PbO fly ash, and chrysotile asbestos were decreasingly active below the F-Met-Phe latex, the chrysotile being about one-half as active as the fumed silica. Rock dust and road dust stimulated to levels slightly higher than

TABLE I
COMPARISON OF PARTICLE EFFECTS ON MAXIMAL OXIDANT PRODUCTION BY ALVEOLAR MACROPHAGES

Particle	Maximal chemiluminescence ($V/10^6$ cells)[a]
Crocidolite asbestos	4.68 ± 1.25 (11)
F-Met-Phe-washed latex (1.1 μm)	2.61 ± 0.37 (4)
Fumed silica	2.12 ± 0.83 (6)
F-Met-Phe-washed glass beads (3–9 μm)	1.93 ± 0.68 (4)
Silicic acid	1.72 ± 0.47 (6)
Zymosan	1.63 ± 1.01 (7)
Polymethyl methacrylate microspheres (0.33 μm)	1.58 ± 0.24 (5)
PbO fly ash	1.26 ± 0.55 (10)
Crysotile A asbestos	1.14 ± 0.15 (7)
Rock dust	1.09 ± 0.44 (6)
Road dust	0.92 ± 0.33 (6)
Glass beads (3–9 μm)	0.73 ± 0.29 (4)
Latex microspheres (1.1 μm)	0.72 ± 0.48 (12)
Polybead carboxylate microspheres (1.4 μm)	0.50 ± 0.21 (5)
Fly ash, uncoated (2–5 μm)	0.28 ± 0.08 (9)
Fiberglass	0.17 ± 0.02 (9)
Basal	0.18 ± 0.13 (16)

[a] Mean ± SD are shown for the number of animals in parentheses.

glass and latex beads. Polybead carboxylate microspheres, and uncoated fly ash had very low levels of activity, but they had significant elevating effects over the basal level in most cell preparations. Experiments in which cells were placed in different types of vials suggested that the basal levels of oxidant production were due to interaction of the cells with the vials.

Effects of particles and particle supernatants on macrophage responses to a soluble peptide. None of the supernatants obtained by micropore filtration of the particle suspensions had noticeable effects on the basal levels of AM chemiluminescence. Their effects were also tested on AM chemiluminescence stimulated by F-Met-Phe. The only supernatants which affected the F-Met-Phe response were those from amosite asbestos and from NiO- and MnO_2-coated fly ash. These supernatants caused increases in the subsequent F-Met-Phe response of 80, 230, and 51% over control values, respectively.

Also examined were the effects of the particles on the immediate and long-term responsiveness of the cells to F-Met-Phe. Figure 4 shows that fiberglass, zymosan, rock dust, and road dust had little effect on the responsiveness of AM to F-Met-Phe even after 3 hr of incubation with these particles. PbO fly ash and silicic acid had no immediate effect but produced a rapid decline in responsiveness to less than 50% of control values after 3 hr. Uncoated fly ash produced an immediate quenching of F-Met-Phe chemiluminescence to 40% of control values, then only a slight decline in the response thereafter.

All of the forms of asbestos, as well as rock dust and latex, caused an enhancement of the F-Met-Phe response of the AM (Figs. 4A, B). When added immedi-

ately before F-Met-Phe, these particles caused rapid increases in the F-Met-Phe response to as high as twice the usual F-Met-Phe response. As the cells were incubated with the particles for longer periods, their F-Met-Phe response lessened to varying degrees depending on the particle added.

The role of macrophage oxidant production in the cytotoxicity of particles. Alveolar macrophages were incubated with different particles in the presence and absence of the oxidant scavenging enzyme superoxide dismutase (Fig. 5). The concentration of superoxide dismutase that completely inhibited the particle-induced oxidant response (10 μg/ml) had no protective effect on AM exposed to latex, fly ash, PbO fly ash, anthophyllite asbestos, or silicic acid. The time-averaged effects of superoxide dismutase showed significant protection only in untreated cells and in cells stimulated with 10 μM F-Met-Phe.

DISCUSSION

Air pollutant dusts as well as chemically defined particles were tested for their ability to stimulate oxidant production in guinea pig alveolar macrophages. All particles examined were found to stimulate the AM in a dose-dependent manner, but the particles varied greatly in their maximum ability to cause oxidant production. The relative effects of the particles studied were very reproducible although the absolute responsiveness of the AM varied between different guinea pigs. These results add to the previously reported increases in oxidant formation in human, rabbit, mouse, and guinea pig AM stimulated by surface-active agents, latex, or microbial particles (Beall *et al.* 1977; Allen and Loose, 1976; Hoidal *et al.*, 1978; Drath and Karnovsky, 1975; Lowrie and Aber, 1977; Rister and Baehner, 1977; Miles *et al.*, 1977; Ziprin, 1978). Some workers, however, failed to find increased O_2^- production in phagocytizing guinea pig and rabbit AM (Drath and Karnovsky, 1975; DeChatelet *et al.*, 1975). These discrepancies are probably due to differences in the assay procedure for oxidant production.

Three types of amphibole asbestos (crocidolite, anthophyllite, and amosite) were the most active of all the agents studied in our assay, while the serpentine forms (chrysotiles A and B) were about one-fourth as active. Fiberglass insulation, which was the only other fibrous material studied, had almost no stimulatory activity for AM (Fig. 2, Table 1). Different types of UICC asbestos samples have been compared previously in terms of their cytotoxicity for mouse peritoneal macrophages, their hemolytic activity for erythrocytes, and their ability to induce mesotheliomas in rats after intrapleural inoculation (Harington, 1976; Wagner and Berry, 1973). Chrysotiles A and B were reported to be more toxic for macrophages and more hemolytic for erythrocytes than the amphibole asbestos samples, possibly because of the higher magnesium:silicon ratio of the chrysotile versus the amphibole asbestos. The ability of the asbestos samples to cause macrophage oxidase activation in the present study appeared to follow a pattern opposite to the pattern of *in vitro* cytotoxicity; e.g., the amphibole asbestos samples appeared to be more effective than chrysotiles A and B. Wagner and Berry (1973) reported a higher activity for amphibole asbestos samples in the production of mesotheliomas in rats, and an almost complete inactivity of fiberglass in the same system (Wagner *et al.*, 1976). We are as yet unable to determine why such

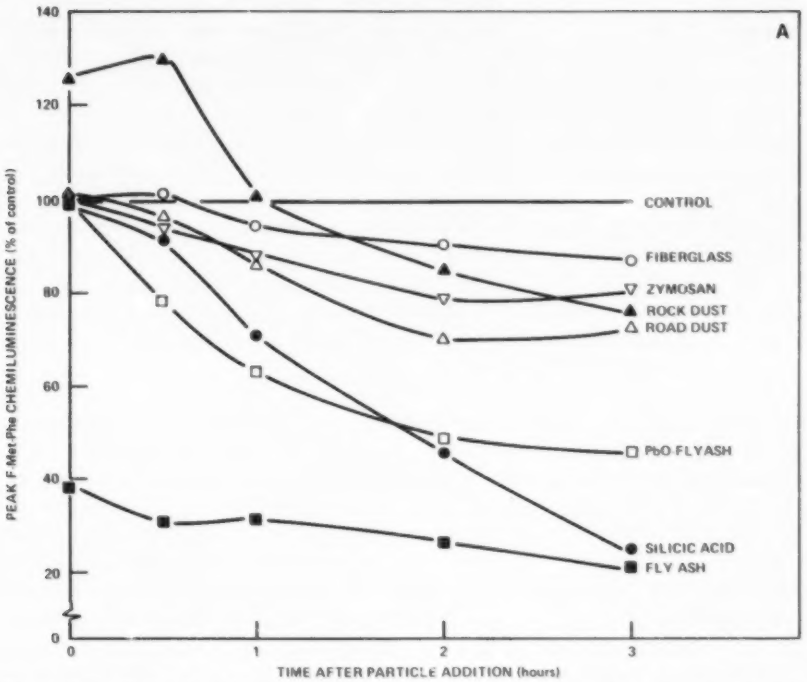

FIG. 4. The effects of particles added at time zero on subsequent maximal response of alveolar macrophages to 10 μM F-Met-Phe. Each point is calculated from the mean of six determinations from three animals. (A) Effects of 1 mg/ml concentrations of fiberglass, zymosan, rock dust, road dust, PbO fly ash, silicic acid, and fly ash on F-Met-Phe responsiveness. (B) Effects of latex (1.1 μm) and different types of UICC asbestos on subsequent responsiveness to F-Met-Phe.

large differences exist between amphibole and chrysotile asbestos in activation of the macrophage oxidase. Microscopic examination of the cells suggests that differences in attachment of the fibers to the cells may be important. Also, metal ions which may be present in greater quantities in the amphibole asbestos might cause an artificially high oxidant response by catalyzing oxidation of the luminol. Luminol at neutral pH values is not spontaneously oxidized by metal ions; however, we have found that Fe^{2+}, Ni^{2+}, and Mn^{2+} but not Pb^{2+} enhance chemiluminescent oxidation of luminol by the xanthine oxidase reaction and by AM stimulated with F-Met-Phe. Catalysis by free metal ions may be responsible for the enhancement of the F-Met-Phe response by supernatants from amosite asbestos. While no enhancing effects were found for other asbestos supernatants, it is possible that metals complexed within the asbestos might also enhance luminol oxidation.

The order of stimulatory activity of the various metal oxide-coated fly ash samples in causing AM oxidant production (PbO > NiO > MnO_2 and uncoated) was similar to their relative cytotoxicity reported previously for rabbit AM and confirmed by our experiments (Fig. 5; Aranyi *et al.*, 1977). The smaller particles,

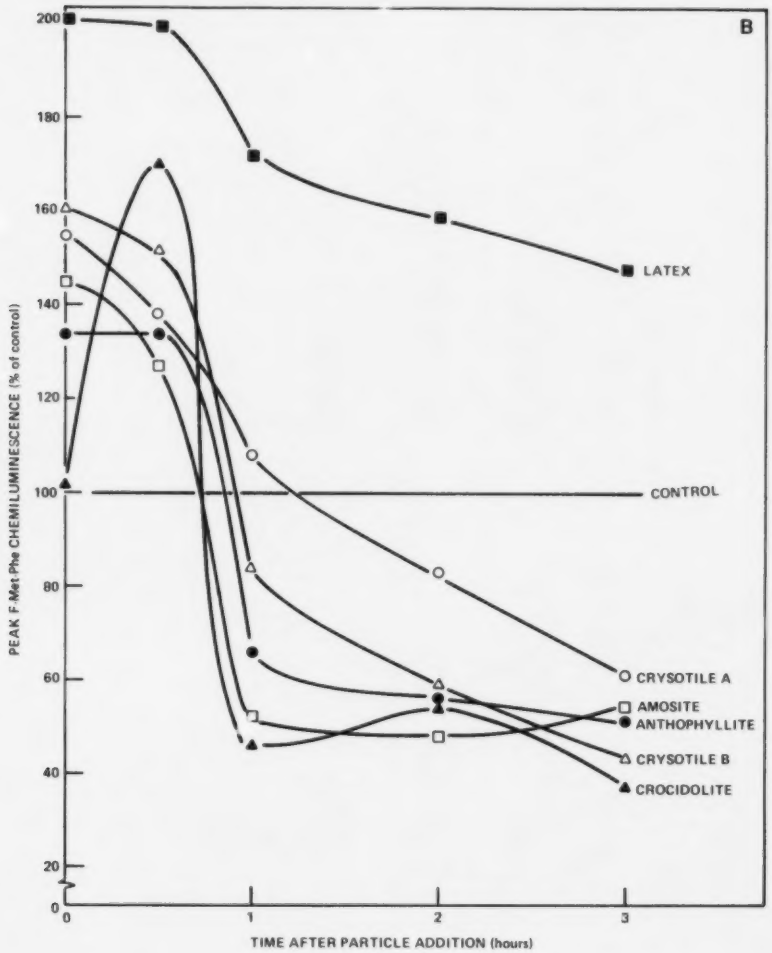

FIG. 4. (*Continued*)

which were previously shown to be more cytotoxic, induced higher levels of oxidant production, with the exception of the PbO-coated fly ash. The enhancing effects of NiO and MnO_2^- fly ash supernatants on the F-Met-Phe-induced response of the macrophages may be attributable to the presence of the free metal ions. Amphoteric metal oxides appear to form increasing amounts of free metal ions as solutions are made more acidic (Mortimer, 1967), and there may have been sufficient amounts (μM to mM) of the free metal ions to account for the enhancing effect of the supernatants.

It is not yet known what is the cellular stimulus for activation of the macrophage

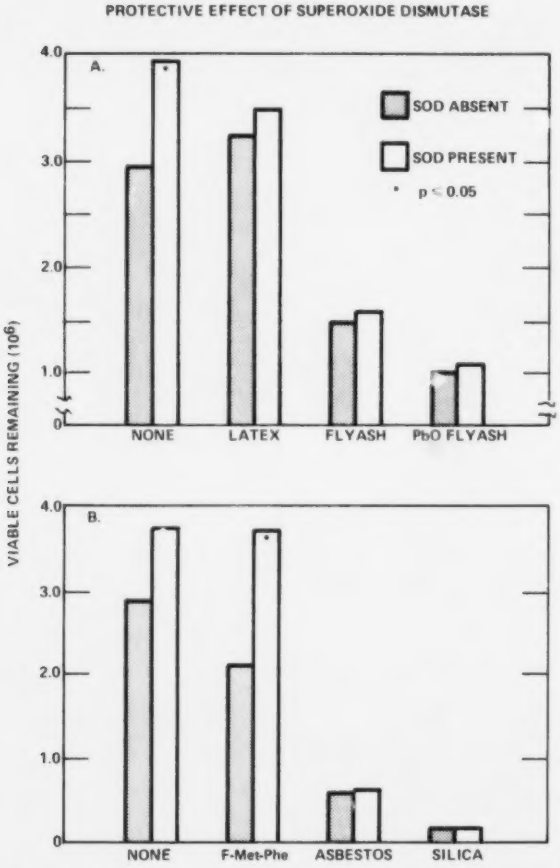

FIG. 5. The effect of added superoxide dismutase on viability of alveolar macrophages exposed to particles. Viability determined by trypan blue exclusion was performed in triplicate, each sample from a different animal. Values are the average of viability determinations performed at 1 and 22 hr after addition of 1 mg/ml of particles or 10 μM F-Met-Phe. The superoxide dismutase concentration was 10 μg/ml.

oxidase. All phagocytosable particles appear to stimulate oxidant production. We have previously shown that soluble peptides also stimulate this mechanism through what appears to be a specific receptor interaction (Hatch *et al.*, 1978). In the present study, the adsorption of F-Met-Phe on the surface of glass and latex enhanced the response of the cells to these particles, suggesting that receptor interactions may contribute to the stimulatory effect of particles. Asbestos appears to mimic microbial cell wall material in its ability to activate complement and to stimulate selective release of lysosomal enzymes from macrophages (Davies *et al.*, 1974; Wilson *et al.*, 1977). It is possible that the enhanced stimula-

tory activity of asbestos is due to its ability to activate the specific receptors of the AM. Silica, however, possesses neither the ability to activate complement nor lysosomal enzyme secretion yet it is an active stimulant of oxidant production. The importance of small differences in the chemistry or surface charge of the particles is illustrated by the large difference in activities of polymethyl methacrylate beads, which possess esterified carboxyl groups, and carboxylate-containing beads, which possess free carboxyl groups on their surface (Table 1). Despite the importance of the particle surface, surface contact alone does not appear to us to be the primary stimulus to oxidant production. The chemiluminescent response to particles follows a more prolonged time course than the attachment of the particles to the cells as observed microscopically, suggesting that the particles require ingestion in order to stimulate the response. In addition, oxidant production by the cells did not appear to be altered as much as would be expected when the surface area of the particles added was varied. Examination of Fig. 1 shows that if one excludes the 7.6-μm particles, the particles ranging from 0.1 to 3.2 μm gave responses which varied by only a factor of 2 in spite of the fact that 60 times more surface area was exposed on the 0.1-μm particles than on the 3.2-μm particles. Moreover, the number of particles per milliliter was 200,000-fold higher with the smaller particles. Particle ingestion in leukocytes has been reported to follow a similar pattern. The volume of uptake of latex remains constant even though the particle size is varied (Roberts and Quastel, 1963). When chemiluminescence was expressed on the basis of the milligrams per milliliter concentration of particles added, the smaller latex and fly ash particles were usually more active than the larger particles (Figs. 1 and 3). These differences due to particle size were, however, slight compared to the differences seen between particles of different composition.

Efforts to show that oxidant production mediates the toxic effects of fly ash and other particles were unsuccessful (Table 2). While superoxide dismutase had a protective effect on resting cells and on cells stimulated with F-Met-Phe, it did not appear to protect AM from the toxic effects of asbestos, silica, and fly ash. Salin and McCord (1975) noted a protective effect of superoxide dismutase and of catalase on human PMN ingesting *E. coli*. The toxic effects they observed due to the phagocytosis of this bacteria appeared to be less severe than effects we have observed in AM due to the action of pollutant particles. Thus, it is possible that the mild toxicity induced by microbial particles and F-Met-Phe is largely due to oxidant radicals, while the more severe toxicity caused by asbestos and silica is not mediated by oxidants. Another possibility is that the oxidative reactions induced by particles occur at sites not accessible to superoxide dismutase. Macrophages may also possess unusually effective protective mechanisms against their own oxidants.

Macrophage-derived oxidants may have other actions not related to cytotoxic effects. Bhatnagar (1977) has proposed a role for superoxide radical in the pulmonary fibrosis accompanying inflammatory conditions, as well as that seen in paraquat and ozone exposure. He has shown that superoxide can replace ascorbic acid as a cofactor in collagen synthesis, and that superoxide serves as both a

cofactor and an inducer for the activity of prolyl hydroxylase, a key enzyme in collagen synthesis.

Stimulation with F-Met-Phe immediately after addition of the particles was used to provide an estimate of the direct light quenching or oxidant scavenging effects of the particles. By this criterion, uncoated fly ash was the only particle that appeared to possess light quenching or oxidant scavenging properties. An unexpected finding was that all asbestos samples, as well as rock dust and latex, caused an enhancement of the subsequent F-Met-Phe response which lasted for approximately 1 hr. This phenomenon did not appear to be caused by additivity of the particle-induced response to that of F-Met-Phe because it occurred before the normal cellular response to the particles. Light scattering or catalysis of luminol oxidation by the particles do not seem to be involved since the enhancement disappeared with time more rapidly than the particles and fibers were removed by phagocytosis. A possible explanation for this enhancement is that particle binding with the membrane causes partial immobilization of membrane F-Met-Phe receptor sites and the oxidase so that interaction of the two is more likely to occur. The binding of F-Met-Phe or luminol–BSA to the particles could further potentiate this effect. All of the cells, when incubated with the particles, became less responsive through time after addition of the particles. The rate of decline in the responsiveness of the cells to F-Met-Phe was greater after addition of the more cytotoxic particles; however, decreased responsiveness also occurred in viable cells during culture or after addition of nontoxic particles. Incubation for 22 hr, for example, decreased AM viability in a representative experiment by 10%, while the responsiveness to F-Met-Phe was decreased by 79%. Experiments are now being performed to determine under what conditions the decreased responsiveness to F-Met-Phe is correlated with decreased viability, as well as to determine the biological significance of the loss of responsiveness to F-Met-Phe in viable cells. The technical difficulty of performing accurate viability determinations in particle-laden cells underlines the need for an alternative measurement of the health of AM exposed to particles.

The production of oxidants by macrophages, an activity which can be stimulated by pollutant particles, may be an important variable in determining the fate of oxidizable substances adsorbed to particles. Further investigations will be necessary to determine the effects of macrophage-initiated oxidative reactions on biologically important compounds and the mechanism of these reactions, and to characterize further the factors which determine macrophage-stimulating properties of particles.

ACKNOWLEDGMENTS

The authors wish to thank David N. Drechsel, Bruce L. Henschen, and Dianne S. Litaker for technical assistance.

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